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Role of Anti-gB and -gD Antibodies in Antibody-Induced Endocytosis of Viral and Cellular Cell Surface Glycoproteins Expressed on Pseudorabies Virus-Infected Monocytes

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The addition of porcine pseudorabies virus (PrV)-specific polyclonal IgG antibodies to PrV-infected monocytes induces internalization of plasma membrane-anchored viral glycoproteins and major histocompatibility complex (MHC) class I. Using PrV deletion strains, it was shown that gB and gD are essential for the process to occur. The purpose of the current study was to evaluate whether antibodies directed against single viral glycoproteins are able to induce endocytosis. It was shown that monoclonal antibodies directed against viral glycoprotein gB and gD, but not against gC and gE, are able to induce internalization of their respective ligand. Adding a combination of monoclonal antibodies against gB and gD resulted in endocytosis levels, comparable to the endocytosis levels observed when adding porcine PrV-specific polyclonal antibodies. The addition of genistein and tyrphostin 25, two inhibitors of tyrosine kinase activity, abolished endocytosis induced by monoclonal anti-gB and -gD antibodies in a concentration-dependent manner. The addition of similar concentrations of tyrphostin 1, an inactive tyrphostin, had no effect on endocytosis. It was also shown that a mixture of polyclonal, but not monoclonal, antibodies against gB and gD is able to induce cointernalization of MHC class I. This indicates that MHC class I cointernalization results from a passive catching of the molecules rather than from a specific interaction of the MHC class I molecules with one or more viral glycoproteins. In conclusion, it can be stated that antibody-induced crosslinking of gB and gD induces the activation of a tyrosine phosphorylation-dependent signal transduction pathway, leading to their endocytosis. Cointernalization of other viral glycoproteins and MHC class I is most likely caused by a passive catching of these molecules in the gB and gD aggregates.

INTRODUCTION

Herpesviruses are known to have developed several immune evasion strategies (reviewed in York, 1996; Farrell and Davis-Poynter, 1998; Johnson and Hill, 1998). Recently, we described a potential new immune evasion mechanism used by pseudorabies virus (PrV). We showed that the addition of anti-PrV polyclonal antibodies to PrV-infected monocytes induced aggregation and internalization of all major plasma membrane-anchored viral glycoproteins (Favoreel *et al.,* 1999a), leaving viable infected monocytes without visually detectable levels of viral glycoproteins on their plasma membrane. Furthermore, we showed that the majority of the cellular proteins, including major histocompatibility complex class I (MHC class I), are coendocytosed during this process, which may cause an impaired cytotoxic T-lymphocyte recognition of the infected cells.

Interestingly, monocytes infected with PrV strains carrying deletions in the genes encoding glycoproteins B (gB) or gD lost the capacity to internalize their cell surface-bound viral glycoproteins after antibody addition. Monocytes infected with PrV strains that are unable to express one of the remaining nine glycoproteins (gC, gE, gI, gG, gH, gK, gL, gM, gN) did not have an impaired endocytosis capacity, although a PrV strain that lacked both the genes encoding gE and gI had a reduced, but not abolished, endocytosis capacity.

The purpose of the current study was to better understand the role of the different viral glycoproteins during the process by determining whether antibodies directed against single viral glycoproteins are able to induce endocytosis. We show that monoclonal antibodies directed against gB and gD, but not monoclonal antibodies against gC and gE, trigger a tyrosine phosphorylation-dependent signal transduction pathway, which leads to internalization of the respective ligands. Furthermore, we show that the addition of polyclonal, but not monoclonal, anti-gB and -gD antibodies results in cointernalization of the majority of the viral and cellular plasma membrane proteins, including gC, gE, and MHC class I.

RESULTS

Monoclonal antibodies against gB and gD induce endocytosis of their respective ligands

The addition of porcine polyclonal anti-PrV antibodies to PrV-infected monocytes induces internalization of all major viral glycoproteins that become expressed on the plasma membrane of the infected cell. Viral glycopro-

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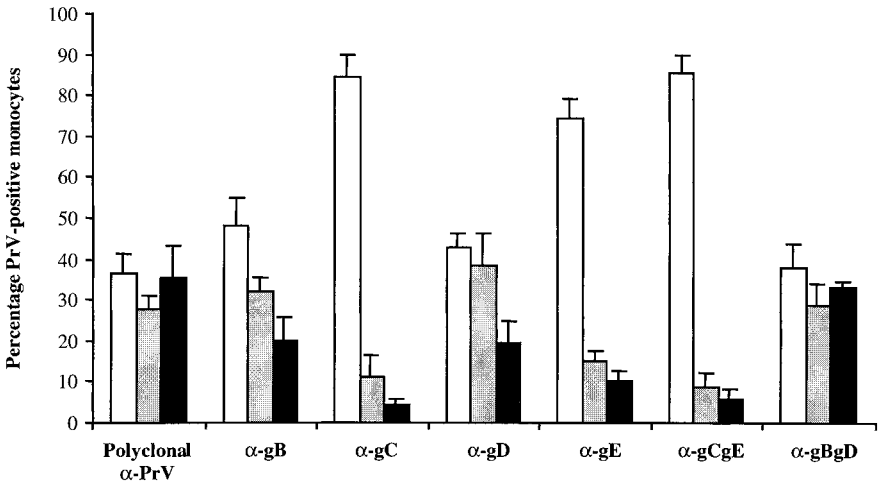


FIG. 1. Monoclonal anti-gB and -gD antibodies induce endocytosis of their respective ligand. PrV-infected monocytes were incubated with porcine polyclonal anti-PrV IgG-FITC or monoclonal antibodies directed against different viral glycoproteins (gB, gC, gD, or gE) for 2 h at 37°C. Then cells were paraformaldehyde fixed, permeabilized, and incubated with FITC-labeled goat anti-mouse IgG. Percentage of cells with patched (open columns), partially endocytosed (gray columns), and completely endocytosed (black columns) viral glycoproteins was counted. Values represent mean + SD of triplicate assays.

teins gB and gD are essential for this process to occur (Favoreel *et al.,* 1999a). To evaluate whether antibodies against single viral glycoproteins are able to induce endocytosis, we added monoclonal antibodies directed against major viral glycoproteins gB, gC, gD, and gE to PrV-infected monocytes. Figure 1 shows that only monoclonal antibodies against gB and gD are capable of inducing endocytosis. The percentage of endocytosis observed by the addition of antibodies against gB and gD is lower than when using anti-PrV polyclonal antibodies. However, when adding a mixture of monoclonal antibodies against gB and gD, the level of endocytosis is comparable to the level observed when using polyclonal anti-PrV antibodies.

The different monoclonal antibodies have been used in comparable concentrations of IgG (0.05 mg/ml). However, the inability of the antibodies against gC and gE to induce endocytosis may be explained by a lower affinity of the antibodies to their respective viral glycoproteins or by a significant lower cell surface expression of gC or gE compared with gB or gD. To evaluate this possibility, monocytes were mock or PrV infected for 13 h, paraformaldehyde fixed, and incubated with the different monoclonal antibodies at the same concentration as used for the endocytosis experiments. Then cells were washed and incubated in 1:50 FITC-labeled goat anti-mouse antibodies (Molecular Probes, Eugene, OR) for 1 h at room temperature, and the fluorescence intensity of the cells was analyzed by flow cytometry. As can be seen in Fig. 2, there is no significant difference between the different antibodies in affinity for their respective viral glycoproteins, nor is there a significant difference in level of plasma membrane expression between the different major viral glycoproteins.

The observation that the level of plasma membrane expression of gB, gC, gD, and gE is comparable at 13 h postinoculation is in agreement with earlier studies (Nauwynck, 1993).

To demonstrate that the low levels of endocytosis observed using anti-gC and -gD antibodies could not be overcome by using higher concentrations of antibodies, we repeated the endocytosis experiments using 0.2 mg/ml IgG. This resulted in total endocytosis percentages (addition of percentages of partial and complete endocytosis) of 48.1% for anti-gB antibodies, 11.0% for anti-gC antibodies, 48.0% for anti-gD antibodies, and 17.9% for anti-gE antibodies. These results are comparable to the results obtained by using 0.05 mg/ml IgG (Fig. 1).

Endocytosis induced by the addition of anti-gB and -gD antibodies is dependent on tyrosine kinase activity

Bivalent ligand-induced endocytosis of cellular receptors is often dependent on a tyrosine phosphorylation signalling pathway (Sibley *et al.,* 1987). To demonstrate that crosslinking of gB and gD by the addition of anti-gB and -gD monoclonal antibodies also initiates a tyrosine phosphorylation-dependent signalling pathway, two tyrosine kinase activity-inhibiting reagents, genistein and tyrphostin 25 (Sigma Chemical Co., St. Louis, MO), were added at different concentrations (0–50 μg/ml) to the PrV-infected monocytes 45 min before and during antibody incubation. None of the reagents used had any effect on cell viability or viral glycoprotein patching. Figure 3 shows that these reagents are capable of inhibiting antibody-induced endocytosis in a concentration-dependent manner. Furthermore, the addition of an inactive form of tyrphostin, tyrphostin 1 (Sigma Chemical Co.), at similar concentrations did not have any effect on antibody-induced endocytosis (Fig. 3).

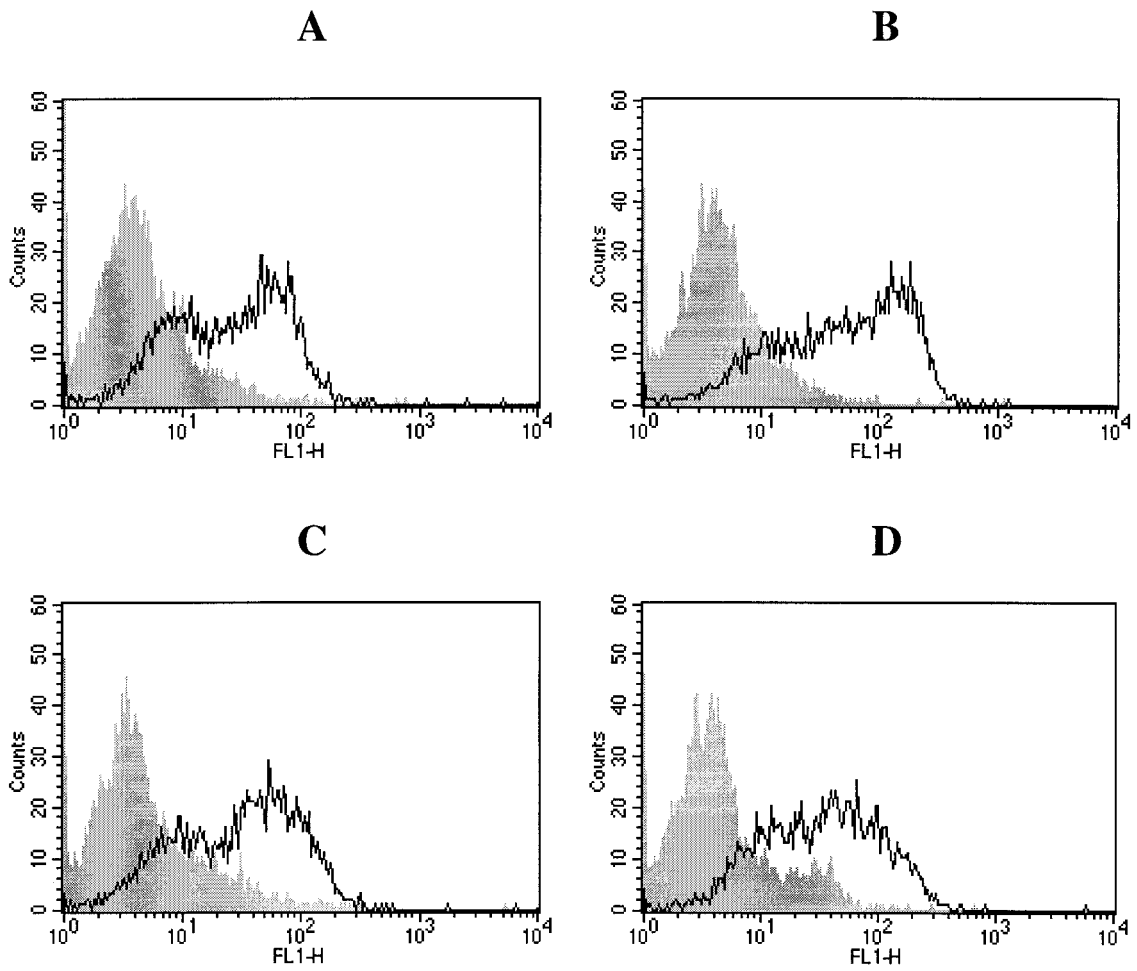


FIG. 2. Plasma membrane expression of different major viral glycoproteins and the affinity of the different monoclonal antibodies is comparable. PrV-infected (solid lines) or mock-infected (shaded plots) monocytes were paraformaldehyde fixed and subsequently incubated with monoclonal antibodies (0.05 mg/ml IgG) against gB (A), gC (B), gD (C), or gE (D) and FITC-labeled goat anti-mouse antibodies. Fluorescence intensity was analyzed by flow cytometry.

Polyclonal, but not monoclonal, antibodies against gB and gD induce coendocytosis of most plasma membrane proteins, including gC, gE, and MHC class I

The addition of PrV-specific polyclonal antibodies induces endocytosis of all major viral glycoproteins and most cellular proteins as well. To evaluate whether the addition of monospecific antibodies against gB and gD also results in cointernalization of other major viral glycoproteins and certain cellular proteins, a series of immunofluorescence doublestainings were performed.

To evaluate whether the majority of plasma membrane proteins is cointernalized, PrV-infected cells were biotinylated on ice for 1 h, washed, and incubated at 37°C in the presence of monoclonal or polyclonal antibodies against gB and gD. After 1 h, the cells were fixed, permeabilized, and doublestained with FITC-labeled secondary antibodies and Texas Red-linked streptavidin.

As seen in Fig. 4, monoclonal anti-gB and -gD antibodies are unable to induce coendocytosis of other plasma membrane proteins. However, the addition of polyclonal antibodies against gB and gD results in the cointernalization of several other plasma membrane proteins.

To evaluate whether major viral glycoproteins (gC, gE) and the immunologically important MHC class I are among these internalized proteins, PrV-infected cells were incubated with polyclonal anti-gB and -gD antibodies, paraformaldehyde fixed, permeabilized, incubated with mouse anti-gC (or -gE or -MHC class I), and subsequently stained with a mixture of FITC-labeled rabbit anti-swine antibodies and Texas Red-linked goat anti-mouse antibodies. Figures 5a–5c show that viral glycoproteins gC and gE and the cellular protein MHC class I undergo cointernalization after the addition of polyclonal anti-gB and -gD antibodies.

We determined whether the capability of the polyclonal anti-gB and -gD antibodies to induce coendocytosis can be explained by the capacity of these antibodies to induce antibody bipolar bridging. This involves simultaneous binding of the antibodies to the viral antigens with their Fab domain and to Fc receptors present on the

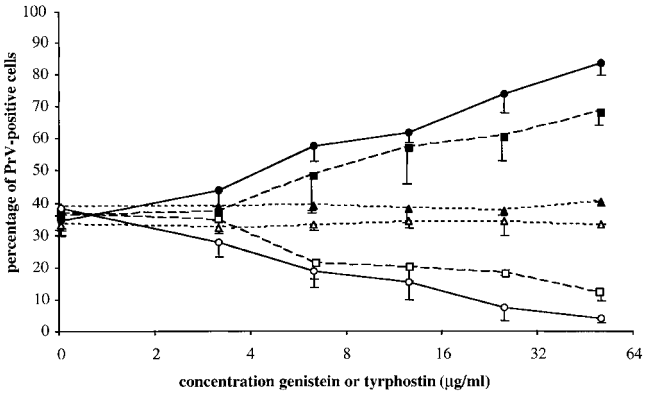


FIG. 3. gB- and gD-mediated endocytosis is tyrosine phosphorylation dependent. PrV-infected monocytes were incubated with different concentrations of the tyrosine kinase inhibiting genistein (solid lines) or tyrphostin 25 (dashed lines) or the inactive tyrphostin 1 (dotted lines) for 45 min at 37°C before and during the incubation of the monocytes with a mixture of monoclonal anti-gB and -gD antibodies for 2 h at 37°C. Then cells were paraformaldehyde fixed, permeabilized, and incubated in FITC-labeled goat anti-mouse antibodies. Percentage of monocytes displaying patched (filled symbols) or completely endocytosed (open symbols) viral glycoproteins was determined. The data are mean ± SD of triplicate assays.

plasma membrane with their Fc domain. Because swine antibodies (and the relatively closely related goat antibodies; Aida *et al.,* 1992) have a higher affinity for swine Fc receptors than mouse antibodies, they are more likely to induce antibody bipolar bridging (Rasmussen *et al.,* 1983; Johansson *et al.,* 1985). To evaluate the possible importance of Fc receptors for cointernalization, we blocked them by preincubating PrV-infected cells with 0.5 mg/ml PrV-negative swine IgG 45 min before and during incubation of the monocytes with polyclonal anti-gB and -gD antibodies. However, this preincubation did not abolish or reduce cointernalization of the other plasma membrane proteins (data not shown).

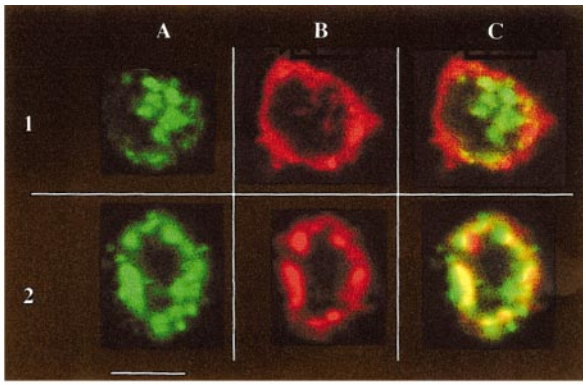
DISCUSSION

In the present report, we showed that crosslinking of PrV glycoproteins gB and gD by the addition of monoclonal anti-gB and -gD antibodies to PrV-infected monocytes induces a tyrosine phosphorylation-dependent signal transduction pathway, which ultimately leads to internalization of gB and gD. Monoclonal antibodies against gC and gE did not induce endocytosis. We showed that the inability of antibodies against gC and gE to induce endocytosis cannot be explained by a lower affinity of the antibodies to the respective viral glycoproteins or by a lower plasma membrane expression of gC or gE compared with gB or gD. Furthermore, we showed that polyclonal, but not monoclonal, antibodies against gB and gD induce cointernalization of the majority of the plasma membrane proteins, including gC, gE, and MHC class I.

These results stress the importance of viral glycoproteins gB and gD during antibody-induced endocytosis of viral cell surface glycoproteins on PrV-infected monocytes. Earlier experiments using PrV strains carrying deletions in the genes coding for different viral glycoproteins (Favoreel *et al.,* 1999a) showed that both glycoproteins are essential for the process to occur. In these earlier studies, it was also shown that deleting the gE–gI complex (but not gE or gI alone) results in a significant decrease in endocytosis (Favoreel *et al.,* 1999a). Because deleting either gE or gI alone has no effect on antibody-induced endocytosis, we did not expect the binding of specific antibodies to gE or gI to be important in the role of the gE–gI complex during endocytosis. The current data support this hypothesis, because gE-specific antibodies do not induce endocytosis. The function of the gE–gI complex during endocytosis therefore remains to be studied, especially the possible role of its Fc receptor activity in the process.

The present data provide further evidence that antibody-induced endocytosis of viral glycoproteins is closely related to bivalent ligand-induced endocytosis of cellular receptors. The mechanism of receptor endocytosis on crosslinking is quite well understood. Crosslinking of such a receptor (by the addition of bivalent ligands or antibodies) induces the binding of adaptor proteins to its cytoplasmic tail. This binding is dependent on some highly conserved tyrosine-based amino acid sequences in the cytoplasmic tail of the receptor (Ohno *et al.,* 1995, 1996; Heilker *et al.,* 1996). The consensus sequence for these tyrosine-based motifs is YxxPhi (where Y stands for tyrosine, x for any amino acid, and Phi represents a bulky hydrophobic residue, such as leucine, isoleucine, phenylalanine, methionine, or valine). The adaptor proteins link the receptors with clathrin molecules, which are the driving force behind membrane invagination by the formation of clathrin-coated vesicles (Shih *et al.,* 1995; Heilker *et al.,* 1996; Hirst and Robinson, 1998). Tyrosine phosphorylation of the clathrin molecules appears to be necessary for the invagination (Goren *et al.,* 1991; Mooibroek *et al.,* 1992). Several downstream events in the endocytosis process are also dependent on tyrosine phosphorylation (Sibley *et al.,* 1987).

In the current report, we show that at least some of the initial steps in gB- and gD-mediated antibody-induced endocytosis show interesting analogies to receptor internalization. Preceding crosslinking of gB and/or gD is necessary for endocytosis to occur, as well as the activation of tyrosine kinases, because genistein and tyrphostin 25 (but not the inactive tyrphostin 1) inhibit the endocytosis process. Furthermore, it is intriguing that viral glycoprotein gB contains a YMSI-34x-YQRL sequence (I stands for isoleucine) and gD contains a YIFF-7x-YRLL (F stands for phenylalanine) sequence. Future experiments are in progress to construct PrV mutants expressing carboxyl-terminal truncated and point-mutated forms of gB and/or gD. This will provide more indications of whether antibody-induced viral glycopro-



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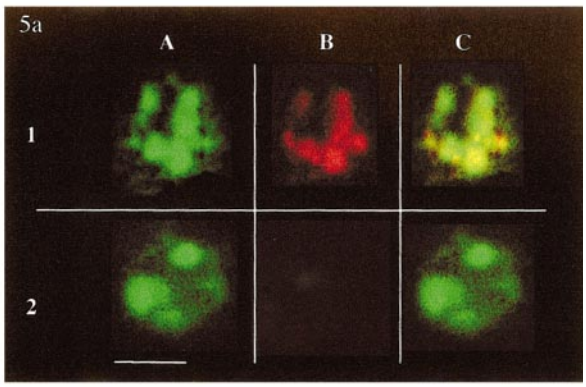

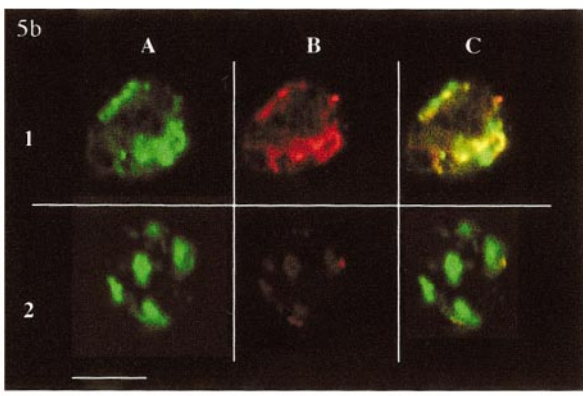

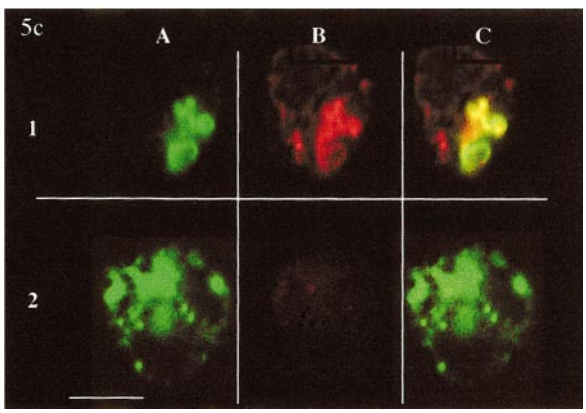

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FIG. 4. Polyclonal, but not monoclonal, antibodies against gB and gD induce cointernalization of the majority of the plasma membrane proteins. PrV-infected monocytes were biotinylated and incubated with a mixture of either monoclonal (1) or polyclonal (2) antibodies for 2 h at 37°C, paraformaldehyde fixed, permeabilized, and incubated with streptavidin-Texas Red and either FITC-labeled goat anti-mouse IgG or FITC-labeled rabbit anti-goat IgG. Distribution of viral glycoproteins (A) and biotinylated proteins (B) was determined by confocal microscopy. Panel C shows the images obtained by merging panels A and B, giving a yellow signal when A and B colocalize. Bar, 10 μm.

FIG. 5. Polyclonal anti-gB and -gD antibodies induce cointernalization of MHC class I, gC, and gE. PrV-infected monocytes were incubated with polyclonal anti-gB and -gD antibodies for 2 h at 37°C, paraformaldehyde fixed, permeabilized, and subsequently incubated with monoclonal antibodies against either MHC class I (a), gC (b), or gE (c) and a mixture of Texas Red-labeled goat anti-mouse IgG and FITC-labeled rabbit anti-swine IgG. In each panel (a–c), lane 1 shows the distribution of the viral glycoproteins in green (A); the distribution of MHC class I (a), gC (b), or gE (c) in red (B); and a merged image of A and B in C. In a–c, lane 2 shows the result of an identical staining as in lane 1 without the monoclonal antibodies against MHC class I (a), gC (b), or gE (c). Bars, 10 μm.

tein endocytosis is some kind of a viral mimicry of cellular receptor endocytosis.

Spontaneous endocytosis of different PrV glycoproteins (gB and gE) has already been described (Tirabassi and Enquist, 1998). However, it is important to clearly discriminate this kind of endocytosis and the antibody-induced endocytosis studied in the current report. Spontaneous endocytosis is restricted to the first 6 h postinoculation (Tirabassi and Enquist, 1998), whereas antibody-induced endocytosis can be induced up to 17 h postinoculation (Favoreel *et al.,* 1999a). However, in the context of our current observations, it remains interesting that spontaneous gE endocytosis is mediated by a single tyrosine residue in one of the two YxxPhi sequences in its cytoplasmic tail (Tirabassi and Enquist, 1999).

Furthermore, we showed earlier that antibody-induced capping and shedding of viral glycoproteins on PrV-infected swine kidney cells are dependent on these two tyrosine residues in the cytoplasmic tail of gE as well as on the activity of tyrosine kinases (Favoreel *et al.,* 1999b). This leads us to the hypothesis that depending on the infected cell type, crosslinking of viral glycoproteins triggers different viral glycoproteins to transduce a signal to the infected cell, with different consequences. The difference in cellular response on viral glycoprotein crosslinking is most probably due to differences in the arsenal of signalling and cytoskeletal proteins between the different cell types. Monocytes are very well equipped to internalize proteins, because their daily function consists of the endocytic and phagocytic uptake of foreign or self material (Allen and Aderem, 1996). Certain epithelium-like cells, more comparable to the swine kidney cells we used, on the other hand, have been reported to shed cellular surface antigens on antibody-induced crosslinking *in vitro* and *in vivo* (although the shedding of viral proteins had not previously been reported) (Camussi *et al.,* 1989; Fukatsu *et al.,* 1989).

Some confusion may rise on the role of gB and gD during antibody-induced endocytosis when comparing the results obtained in this study and the results obtained by using gB- or gD-deleted PrV strains (Favoreel

et al., 1999a). In these studies, we showed that both gB and gD are essential for antibody-induced internalization of viral glycoproteins, because deletion of either one of the proteins resulted in a dramatic decrease in the percentage of cells displaying endocytosis. However, in the current report, we show that monoclonal antibodies directed against either one of the viral glycoproteins are capable of inducing endocytosis, albeit predominantly partial endocytosis. Only the addition of antibodies against both viral glycoproteins resulted in maximal levels of complete endocytosis. Apparently, crosslinking of both viral glycoproteins is necessary for efficient endocytosis. Repeating the earlier experiments using monocytes inoculated with the gB- or gD-mutated PrV strains but incubating them with antibodies for 2 h instead of 1 h showed that approximately 30% of the monocytes are able to induce partial (and sometimes even complete) endocytosis at that time point (compared with 14% at 1 h after the antibody addition; Favoreel *et al.,* 1999a, and unpublished observations). This suggests that crosslinking both gB and gD results in a stronger signal to the cell than crosslinking either one of them separately, which results in a more efficient mobilization of the cellular endocytosis machinery, leading to fast and complete endocytosis.

Furthermore, our studies show that polyclonal, but not monoclonal, antibodies against gB and gD are capable of inducing cointernalization of the majority of the other plasma membrane proteins, including MHC class I, gC, and gE. Preincubation of the cells with PrV-negative IgG did not abolish the capacity of the polyclonal antibodies to induce coendocytosis. Therefore, antibody bipolar bridging, consisting of the simultaneous binding of antibodies to their respective ligand (e.g., gB or gD) with their Fab domain and to Fc receptors on the plasma membrane with their Fc domain, cannot be involved. The most obvious explanation for the observation is that polyclonal antibodies are able to induce cointernalization because they consist of a mixture of antibodies that recognize different epitopes on the same viral glycoprotein. Monoclonal antibodies recognize one specific epitope and are therefore unable to cluster more than two antigens at a time. Polyclonal antibodies recognize a variety of epitopes and are therefore able to produce large aggregates of viral glycoproteins. Other proteins may get passively caught in these large patches and subsequently become internalized.

In conclusion, we can state that the addition of polyclonal anti-gB and -gD antibodies to PrV-infected monocytes clears the plasma membrane of the infected cell from several major viral glycoproteins as well as MHC class I. This suggests that antibody-induced endocytosis of viral and cellular glycoproteins may represent another example of viral mimicry, where the virus misuses a cellular function to its own benefit (e.g., immune evasion).

MATERIALS AND METHODS

Viruses

PrV strain 89V87 [PrV (89V87)] was used for all experiments (Nauwynck and Pensaert, 1992).

Antibodies

Porcine FITC-labeled polyclonal anti-PrV antibodies were described earlier (Nauwynck and Pensaert, 1992; Favoreel *et al.,* 1997). Mouse monoclonal antibodies against gB (1C11), gD (13D12), and gE (18E8) were produced at the laboratory (Nauwynck and Pensaert, 1995). Mouse monoclonal antibody 8P19 against gC was kindly provided by Dr. A. Brun (Mérial, Lyon, France). Polyclonal goat antibodies against gB were kindly provided by Dr. T. Tsuda (Tsuda *et al.,* 1991), and polyclonal swine antibodies against gD were kindly provided by Dr. S. Brockmeier (Brockmeier *et al.,* 1993). Dilutions of the antibodies were made in RPMI 1640 medium (GIBCO BRL, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% FCS, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 1 mM sodium pyruvate, and 1% nonessential amino acids 100× (GIBCO BRL) (medium A).

Isolation of blood monocytes

PrV-negative pigs were used as blood donors at the age of 2.6–6 months. Blood was collected from the vena jugularis on heparin (15 U/ml; Leo, Zaventem, Belgium), and blood mononuclear cells were separated on Ficoll–Paque (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions. Mononuclear cells were resuspended in medium A supplemented with 10 U/ml heparin. Then cells were plated onto 58-mm petri dishes with cell culture coating (Nunc A/S, Roskilde, Denmark) at 2×10^6 cells/ml and cultivated at 37°C with 5% CO_2. After 24 h, nonadherent cells (lymphocytes) were removed by washing the petri dishes three times in RPMI 1640. Purity of the monocytes was assessed by flow cytometric analysis after indirect immunofluorescence labeling of the monocytes through subsequent incubation of the cells in 1:300 74.22.15 (Pescovitz *et al.,* 1984) mouse monoclonal antibody in medium A and 1:100 FITC-labeled goat anti-mouse antibody (Molecular Probes) in medium A. The percentage of monocytes was always ≥75%.

Inoculation of the monocytes

After 24 h of cultivation of the mononuclear cells in petri dishes, lymphocytes were removed as described. PrV (89V87) was added at a multiplicity of infection of 10 in 3 ml of medium A. Cells were further incubated at 37°C with 5% CO_2.

Incubation of PrV-infected monocytes with porcine anti-PrV polyclonal antibodies

Cells inoculated for 13 h were centrifuged at 500 × g for 10 min, washed, and resuspended in medium A. The cells were incubated at 0.6×10^7 cells/ml with 0.25 mg IgG/ml FITC-conjugated PrV polyclonal antibodies for 2 h at 37°C. Every 10 min, the cells were shaken gently to avoid sedimentation. At 2 h after antibody incubation, the cells were fixed with 1% paraformaldehyde for 10 min. Finally, the cells were washed thoroughly, mounted in a glycerin-phosphate buffered saline solution (0.9:0.1, vol/vol) with 2.5% 1,4-diazabicyclo(2.2.2)octane (DABCO; Janssen Chimica, Beerse, Belgium), excited with an Osram HBO 50-W bulb using an I3 filter, and observed with a Leitz DM RBE microscope (Wild Leitz GmbH, Heidelberg, Germany).

Incubation of PrV-infected monocytes with monoclonal or polyclonal anti-gB, -gC, -gD, and/or -gE antibodies

Cells inoculated for 13 h were centrifuged at 500 × g for 10 min, washed, and resuspended in medium A. The cells were incubated at 0.6×10^7 cells/ml with 0.05 mg IgG/ml monoclonal antibodies directed against gB, gC, gD, and/or gE or 0.1 mg IgG/ml polyclonal antibodies directed against gB and gD for 2 h at 37°C. At 60 min after antibody incubation, the cells were fixed with 1% paraformaldehyde for 10 min, washed in PBS, and permeabilized in 0.1% Triton X-100 in FBS (PBS with 20% FCS) for 1 min. Then cells were washed in FBS and incubated for 1 h with either 1:30 FITC-conjugated goat anti-mouse antibodies (Molecular Probes) or 1:30 FITC-labeled rabbit anti-swine antibodies (DAKO A/S, Glostrup, Denmark) in FBS at room temperature. Finally, cells were washed in FBS, mounted in a glycerin-DABCO solution as described earlier, and observed by fluorescence microscopy.

Definition of different viral glycoprotein distributions

The viral glycoprotein distribution was scored as a "rim" when the fluorescence label exhibited a homogeneous cell surface cover. The cells were scored as "patched" when the labeled viral glycoproteins formed randomly distributed aggregates on the cellular surface. Cells with endocytosed vesicles that were not entirely located in the cytoplasm but were still attached to the plasma membrane were scored as "partial endocytosis." When all visible viral glycoproteins were located in vesicles inside the cell, without any remaining on the plasma membrane, cells were scored as "complete endocytosis." The subdivision between partial and complete endocytosis was not made in our former studies (Favoreel *et al.,* 1999a). Hence, when comparing the percentages of endocytosis in both studies, one has to add the percentages of cells with partial and complete endocytosis in the current study to obtain the percentages of endocytosis obtained in the former study.

Quantitative results were obtained by examining the fluorescence distribution on at least 200 cells. The number of cells displaying endocytosis was expressed as a percentage of viral antigen-positive cells. All assays were run independently at least three times.

Biotinylation of plasma membrane proteins and double staining of anti-gB and -gD antibodies and biotinylated plasma membrane proteins

Monocytes inoculated with PrV (89V87) for 13 h were washed twice with cold PBS with Ca^{2+} and Mg^{2+} and incubated with 40 μg biotinylation reagent/10^6 monocytes in 1 ml of cold PBS with Ca^{2+} and Mg^{2+} on ice for 1 h according to the manufacturer's instructions (Amersham International plc, Buckinghamshire, UK). Then cells were washed with cold PBS with Ca^{2+} and Mg^{2+}, resuspended in medium A, and incubated with monoclonal or polyclonal anti-gB and -gD antibodies for 2 h at 37°C before paraformaldehyde fixation and permeabilization as described earlier. After washing in FBS, cells were incubated with either 1:30 FITC-conjugated goat anti-mouse antibodies (Molecular Probes) or 1:30 FITC-labeled rabbit anti-swine antibodies (DAKO A/S) together with 1:30 Texas Red-linked streptavidin (Molecular Probes) in FBS for 1 h at room temperature. Next, cells were washed in FBS, mounted in a glycerin-DABCO solution as described earlier, and observed by confocal microscopy.

Double staining of anti-gB and -gD antibodies and MHC class I, gC, or gE

PrV (89V87)-infected monocytes were incubated with polyclonal anti-gB and -gD antibodies, paraformaldehyde fixed, and permeabilized as described earlier. Cells were washed in FBS; incubated in either 1:100 anti-MHC class I monoclonal antibody (VMRD Inc., Pullman, WA), 1:100 monoclonal anti-gC antibody, or 1:100 monoclonal anti-gE antibody in FBS with 10% goat serum for 1 h at room temperature; washed in FBS with 10% goat serum; and resuspended in 1:30 FITC-labeled rabbit anti-swine antibodies (DAKO A/S), together with 1:100 Texas Red-X-conjugated goat anti-mouse antibodies (Molecular Probes) in FBS with 10% goat serum for 1 h at room temperature. Finally, cells were washed twice in FBS, mounted in a glycerin-DABCO solution as described earlier, and observed by confocal microscopy.

Flow cytometric analysis

Flow cytometric analysis was conducted with a Becton-Dickinson (San Jose, CA) FACScalibur equipped with a 15-mW air-cooled argon ion laser and interfaced to a Macintosh Quadra 650 computer (Apple Computer, Inc., Cupertino, CA) using BD Cellquest software. Acquisition rates were maintained at 300–500 cells/s. At least 5000

cells were analyzed for each sample, and forward-scattered light versus side-scattered light dot plots were used to identify monocyte populations.

Confocal laser scanning microscopy

Fluorescent samples were examined on a Bio-Rad MRC 1024 confocal laser scanning system (Bio-Rad House, Hertfordshire, UK) linked to a Nikon Diaphot 300 microscope (Nikon Corporation, Tokyo, Japan) and interfaced to a Compaq Prosignia 300 computer (Compaq Computer Corporation, Houston, TX). Krypton-argon laser light was used to excite FITC (488-nm line) and Texas Red (568-nm line) fluorochromes. To avoid signal spillover, FITC and Texas Red signals were recorded consecutively with the 488-nm excitation line and the T1 dichroic beam-splitter and the 568-nm excitation line and the T1/E2 beam-splitter, respectively. Extended focus images were obtained with Bio-Rad COMOS and Lasersharp Software.

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